ANTIMICROBIAL SUSCEPTIBILITY OF *ACINETOBACTER* ISOLATES FROM GROOTE SCHUUR HOSPITAL REGION, AN INVESTIGATION OF THE APPROPRIATENESS/VALIDITY OF THE NCCLS ZONE SIZE CRITERIA.

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CONTENTS

FIGURES

TABLES

INTRODUCTION AND AIMS OF THE DISSERTATION:

Since the 1970s, relatively uncommon species of non-enteric gram-negative bacilli have emerged as nosocomial colonizers and pathogens. Among them, one of the most significant genera is *Acinetobacter*, which has given rise to an increasing number of reports of nosocomial infections [9, 13, 25, 41].

The introduction of new broad-spectrum antimicrobials in the hospitals has been one of the main factors responsible for this development. In addition leading to a move from more susceptible towards more resistant pathogens. This occurred both between and within genera [38].

Owing to the unpredictable antimicrobial susceptibility of *Acinetobacter* spp., it is prudent to test each isolate for its susceptibility profile to guide in the proper treatment of infections caused by *Acinetobacter*.

The disk diffusion method is the technique most widely used by microbiology laboratories for the routine assessment of antimicrobial susceptibility patterns. The zone diameters obtained by this technique for individual antimicrobials are reported as susceptible, intermediate or resistant by referring to an interpretative chart. Most laboratories use interpretative charts (zone size criteria) supplied by the National Committee for Clinical Laboratory Standards (NCCLS) for this purpose [31].

In our laboratory we have noted discrepancies between sensitivities as determined by the NCCLS zone size criteria and the local minimum inhibitory concentration (MIC) results obtained for *Acinetobacter* species, especially for cefepime (CPIM) and ceftazidime (CTAZ).

Since *Acinetobacter* species contribute significantly to the increased morbidity and mortality of debilitated patients (especially ventilated ICU patients [19, 25]), and the fact that clinicians rely on the antimicrobial susceptibility results in the management and treatment of these patients, it was felt necessary to investigate the appropriateness/validity of the NCCLS zone size criteria used to interpret the susceptibility test results of cefepime (CPIM), ceftazidime (CTAZ), trimethoprim-sulfamethoxazole (TMP-SULFA), and piperacillin-tazobactam (PIP-TAZO) against *Acinetobacter* spp.

LITERATURE REVIEW:

[A] THE ORGANISM

Acinetobacter spp. are short, plump gram-negative (but sometimes difficult to decolourise) coccobacilli, with a DNA G+C content of 39 to 47 mol%. They appear rod shaped during rapid growth and as coccobacilli during the stationary phase. Cells do not form spores, are generally encapsulated and non-motile (from which they derive their name). They do however display "twitching motility" presumably because of the presence of polar fimbriae. They are strictly aerobic, non-fermentative bacteria that grow well on all common complex media at temperatures between 20°C and 30°C, with most strains having an optimum temperature of 33°C - 35°C. They are oxidase negative and catalase positive.

Acinetobacter is widespread in nature, being found in soil, water and sewage [8, 18].

Taxonomy:

After a colourful history the genus *Acinetobacter* was originally placed in the family *Neisseriaceae*, but was more recently moved to the family *Moraxellaceae*.

Gram-negative, nonfermentative bacteria currently recognized as belonging to the genus *Acinetobacter* have been classified previously under at least 15 different 'generic' names, the best known of which are *Bacterium anitratum*; *Herellea vaginicola; Mima polymorpha; Achromobacter; Alcaligenes; Micrococcus calcoaceticus;* **B5W**; *Moraxella glucidolytica* and *Moraxella lwoffii* [8, 44].

The taxonomy of *Acinetobacter* has changed frequently. Up to now 21 genomic species (DNA-DNA homology groups) have been described. Some of the species have formal names: **group 1** = *A.calcoaceticus*; **group 2** = *A.baumanii*; **group 4** = *A.haemolyticus*; **group 5** = *A.junii*; **group 7** = *A johnsonii*; **group 8** = *A.lwoffii* and **group 12** = *A.radioresistens*, while the rest are unnamed.
There is a close relationship between the genomic species 1 (*A.calcoaceticus*), 2 (*A.baumanii*), 3 and 13 of Tjernberg and Ursing, which are sometimes named as the *A.calcoaceticus-A.baumanii* complex [13].

Clinically the most important species is *A.baumanii*. In one study that included 584 clinical *Acinetobacter* isolates, 72.9% of the isolates were identified as *A.baumanii*, and the remaining 27.1% of the strains isolated belonged to other species, of which the most frequent were *Acinetobacter* genomic species 3 (9.4%), *A.johnsonii* (5.0%) and *A.lwoffii* (6.3%) [13].

Laboratory Identification:

Isolation from clinical specimens.

Acinetobacter spp. can be readily grown on common laboratory media such as nutrient agar. However, for direct isolation from clinical specimens, it is more useful to use a selective medium to suppress the growth of other microorganisms. A selective and differential medium containing bile salts, sugars and bromocresol purple is available commercially as Herellea agar (Difco). A novel antimicrobial-containing selective medium that combines selectivity with differential characteristics has been described, and this medium, Leeds Acinetobacter Medium, is useful for the recovery of most *Acinetobacter* spp. from both clinical and environmental sources [20].

Acinetobacter colonies are smooth, opaque and slightly smaller than those of members of the family *Enterobacteriaceae*. Many strains grow on MacConkey agar as either colourless or slightly pinkish colonies.

All members of the genus are strict aerobes, oxidase negative, and catalase positive and nonfermentative. It is the negative oxidase test that serves as a rapid presumptive test to distinguish *Acinetobacter* spp. from otherwise similar nonfermentative bacteria [39].

Species Identification.

Several different DNA-hybridisation methods can be used to identify the different genomic species, but these methods are laborious and unsuitable for use in the routine microbiology laboratories.

Sixteen of these 21 DNA-homology groups can be differentiated by means of biochemical and growth tests [39]. These phenotypic tests include tests for growth at 30°C, 37°C, 41°C and 44°C, glucose oxidation, gelatin liquefaction, haemolysis on 5% sheep blood agar plates and carbon source assimilation tests.

When referring to strains of DNA groups 1, 2, 3 and 13 of Tjernberg and Ursing, it seems more appropriate at present to use the expression *A.calcoaceticus-A.baumanii* complex, and if there is a need for differentiation within this complex the biotyping system suggested for *A.baumanii* may be used [16].

Simply and practically most glucose oxidizing non-haemolytic clinical strains that can grow at 44°C are *A.baumanii*; most glucose-negative non-haemolytic strains are *A.lwoffii* and most haemolytic strains are *A.haemolyticus* [39].

As far as the commercial identification systems are concerned, the widely used **API 20NE** system, based largely on carbon source assimilation tests contained only *A.baumanii, A.haemolyticus*, and *A.lwoffii* in its 1996 database, together with *A.junii* and *A.johnsonii* as a combination, whereas the type species *A.calcoaceticus* and the other genomic species were not included at all. Problems in respect of sensitivity and reproducibility have been experienced when using this system [24].

Several studies comparing the **API 20NE** system with species identification by DNA-DNA hybridisation have demonstrated a poor correlation [46].

Phenotypic identification methods for individual *Acinetobacter* species are not totally reliable, and this can be a source of confusion in clinical microbiology laboratories. To avoid the problems with phenotypic species identification, new molecular identification methods are currently being evaluated against DNA-DNA hybridisation.

Epidemiology and Pathogenesis:

Epidemiology.

Acinetobacter is widely spread in nature, being found in soil and water and frequently present in the hospital environment. Being non-fastidious and hardy they can survive on moist and on dry surfaces. After experimental contamination of fingers *A.baumanii* survived 60 minutes. Longer survival times were not examined, but on dry Formica surfaces *A.calcoaceticus* survived up to 13 days [13].

Acinetobacter spp. are part of the normal flora of human skin and can be isolated from the axillae, groin, toe webs, and antecubital fossae of about one fourth of the population. Oropharyngeal or rectal carriage is rare. *Acinetobacter* spp. has been found on the hands of 19% of hospital personnel and may be regarded as part of the non-transient flora [17].

Non-transient *Acinetobacter* and other gram-negative bacilli on the hands of hospital personnel are thus a potential reservoir of hospital strains.

In outbreak situations colonization rates of the respiratory tract, the pharynx, the skin, the urinary tract and the gastrointestinal tact of patients may be high. Hand carriage in outbreaks was reported to be transient; transmission of an epidemic strain from the patient skin to the staff member hands has been demonstrated experimentally. A decline in the incidence of hand carriage occurred after reinforcement of strict hand washing procedures [13, 14].

From a study by Allen & Green [4] it was concluded that airborne *Acinetobacter* could produce extensive environmental contamination, and the possibility of airborne spread should be considered in future outbreaks.

In an outbreak of *Acinetobacter* spp. meningitis in leukaemic children it was determined that the methotrexate was extrinsically contaminated by reused needles, used for reconstitution and administration, which had been inadequately sterilized. After the introduction of single-use disposable needles no subsequent cases occurred [22].

In a recent investigation of an outbreak of nosocomial *Acinetobacter* infection, the respiratory and anaesthetic equipment and staff hands were originally suspected of being the source and mode of spread (the most common sources in ICUs) but after hygienic procedures were improved, the outbreak did not come to an end. This suggested that the patients were exposed to multiple sources, and the bedding was suspected, leading to an investigation of feathered pillows. These were found to be contaminated with considerable numbers of *Acinetobacter* spp. Replacement with synthetic pillows and correction of the laundry procedure resulted in a significant reduction of *Acinetobacter* spp. isolations [46].

Contamination of mattresses was also found as the cause for an outbreak of *Acinetobacter* infections in burn patients [40].

The above examples illustrate the ubiquitous nature of *Acinetobacter* spp. and the difficulty of establishing the reservoir of infection in many instances. This survival in the inanimate environment can serve as a reservoir for continuous contamination of hands.
However "hand carriage from contact with infected patients is the most likely mode of transmission, although staff hand contamination from inanimate objects may also be an important factor"[13].

From July 1974 through December 1977 180,982 nosocomial infections were reported to the **CDC** (Center for Disease Control), among these *Acinetobacter* spp. were identified as the pathogen in 1,372 (0.76 %). Even though the overall rates of nosocomial infections caused by *Acinetobacter* spp. were stable each year, there was a dramatic seasonal fluctuation; the rate of infection was twice as high in late summer as in the early winter. The same seasonal increase was observed in medical and surgical services and at all major sites of infection. The reasons for this phenomenon are unclear, but they may be related to increased ambient humidity favorable for this organism's growth [35].

McDonald *et al* [27] also documented this seasonal pattern as observed from 1987 through 1996. But in the **SENTRY** Antimicrobial Surveillance Program (1997-1999) [15], it was noticed that this seasonal pattern was lacking in Latin America. Many reasons could account for the lack of this pattern in Latin America, such as the persistence of endemic strains in some medical centres allowing the occurrence of clustered epidemic *Acinetobacter* infections throughout the year, or an insufficient number of strains collected monthly in the **SENTRY** program to clarify seasonal prevalence.

Typing methods are important tools for establishing the sources and mode(s) of transmission for epidemic strains. There are a number of typing systems, which include: **biotyping**, using biochemical profiles; **antibiograms**, which uses antibiotic susceptibility patterns; **serotyping**; **phage typing**; **bacteriocin typing**; **protein profiles** of both cell wall and whole cell proteins; **multilocus enzyme electrophoretic typing** which investigates the relative mobilities of a large number of cellular enzymes; **plasmid profiles**; **analysis by pulse field gel electrophoresis** of restriction fragment length polymorphism; and **ribotyping**.
No single typing system has so far gained acceptance for typing *Acinetobacter* spp., and this area is still the subject of research [8].

Pathogenesis:

Acinetobacter spp. are generally considered to be relatively non-pathogenic to healthy individuals, but despite this low pathogenic potential they are increasingly reported as the causal organism of numerous hospital outbreaks.

Various risk factors predisposing to severe infection with *Acinetobacter* spp. have been identified. These risk factors include patients who have recently undergone major surgery, those with severe underlying disease, use of antimicrobial agents, presence of invasive devices, and those at extremes of age.

Since the only factor amenable to control in the ICU setting is antimicrobial therapy, avoidance of unnecessary antimicrobials should be a high priority in management of such patients. The use of antimicrobials may alter the normal flora and result in the selection of resistant microorganisms such as *Acinetobacter* spp.

Certain characteristics of *Acinetobacter* spp. may enhance the virulence of strains involved in infections. These characteristics include **(i)** the presence of a polysaccharide capsule; **(ii)** the property of adhesion to human epithelial cells is related to the presence of fimbriae and/or capsular polysaccharide; **(iii)** the production of enzymes which may damage tissue lipids; and **(iv)** the potentially toxic role of the lipopolysaccharide component of the cell wall and the presence of lipid A. The production of endotoxin in-vivo is probably responsible for the disease symptoms observed during Acinetobacter septicaemia [44].

Nosocomial infections:

Acinetobacter spp. have emerged as particularly important organisms in ICUs, and this is probably related to the wide use of antimicrobial agents, and the increasing use of invasive diagnostic and therapeutic procedures in hospital ICUs.

The true frequency of nosocomial infection caused by *Acinetobacter* spp. is not easy to assess, partly because the isolation of these organisms from clinical specimens may not necessarily reflect infection but, rather, may result from colonization [41]. According to data from the National Nosocomial Infections Surveillance (NNIS) system from 1990-1992, *Acinetobacter* spp. were isolated in 1% of all nosocomial infections [13].

The most common site of nosocomial infections caused by *Acinetobacter* spp. is the respiratory tract, especially in ventilated patients, with a crude mortality rate of 30% to 75% reported [9]. This is followed by bacteremia, with *A.baumanii* being the most commonly isolated of the *Acinetobacter* spp. Debilitated patients make up the largest group of adult patients.
A second important group of patients is neonates [32, 37].

Less frequently, the urinary tract, surgical wounds, the central nervous system, the peritoneum, burn wounds, and the eyes are involved [13].
Acinetobacter cholangitis and septic complications following percutaneous transhepatic cholangiogram and percutaneous biliary drainage have been reported [36].

Although community-acquired *Acinetobacter* infections are relatively uncommon, Anstey and co-workers reported that 10% of cases of community-acquired pneumonia in the Northern Territory of Australia were caused by *Acinetobacter* spp. [6].

Antimicrobial susceptibility and Treatment:

The genus *Acinetobacter* is an environmental organism, which seems to have a propensity to develop antimicrobial resistance very rapidly, compared to the more traditional pathogenic bacteria, which require more time to acquire highly effective resistance mechanisms in response to antimicrobial exposure.

Until the early 1970s, nosocomial *Acinetobacter* spp. were amenable to treatment with β-lactams, aminoglycosides and quinolones, either as single agents or in combinations. Since 1975 nosocomial *Acinetobacter* spp., especially *A. baumanii*, have become increasingly resistant to clinically achievable levels of most commonly used antimicrobial agents.

Resistance to the β-lactams is mainly due to the production of β-lactamases [33], in addition to the reduced permeability of the outer membrane proteins of *Acinetobacter* spp. and the low affinity of their penicillin binding proteins.

Aminoglycoside resistance has also been reported, due to altered permeability, lack of intracellular antimicrobial binding sites and to the presence of aminoglycoside modifying enzymes [12].

As a result, the broad-spectrum cephalosporins (cefepime and ceftazidime), fluoroquinolones, carbapenems and amikacin have become the main antimicrobials of empiric choice to treat *A.baumanii* infections. Recently quinolone resistance due to gyr A-mutation has been frequently reported [45], and more recently carbapenem resistant *A.baumanii* are being increasingly reported [2, 10, 43].

This emerging resistance among *Acinetobacter* spp. has made the empiric choice of an effective antimicrobial agent increasingly difficult. In addition to this emerging resistance there are significant differences in the Acinetobacter antibiograms, depending on the species and the region of isolation, i.e. the antibiogram for *Acinetobacter* spp. is unpredictable, hence the necessity for reliable susceptibility testing methods to predict the clinical response.

Numerous reports in the medical and scientific literature have documented the high rates of antimicrobial resistance found in *Acinetobacter* spp. [41].
In our laboratory we have also noticed an increased number of *Acinetobacter* spp. developing resistance to almost all the currently available antimicrobials.
A particular concern has been the increase in the number of nosocomial Acinetobacter isolates found resistant to multiple antimicrobial agents, and the resulting therapeutic problems involved in treating patients with nosocomial infections in the ICUs.
Most of the newly developed antimicrobial agents are effective against gram-positive organisms. The development of new agents directed against gram-negative organisms is extremely important and, in the interim, seeking the best agents among the current and older compounds is the only option [15].

As such, treatment must be individualized depending on the antimicrobial sensitivity results of each isolate in-vitro.

Species other than *A.baumanii*, i.e. *A.lwoffii*, *A.johnsonii*, and *A.junii*, are involved less frequently in nosocomial infection and are generally more susceptible to antimicrobials.

[B] IN-VITRO ANTIMICROBIAL SUSCEPTIBILITY TESTING:

The need to know whether an organism is likely to respond to antimicrobial therapy is as old as chemotherapy itself.

Fleming reported the inhibitory effect of what eventually became known as penicillin on solid media by observing an area of growth inhibition of staphylococcal colonies adjacent to a *Penicillium* contaminant on an agar plate.

This, however, was not the first description of what was eventually termed agar diffusion, as this was first utilized by Beijerinck in 1889 for studying the effect of different auxins on bacterial growth. But Fleming in 1924 introduced the use of the ditch plate technique for evaluating antimicrobial qualities of antiseptic solutions. Reddish modified this technique when he cut wells into the agar and filled the wells with antiseptic solutions. In 1940, Heatly introduced the use of absorbent paper for carrying antimicrobial solutions.

Mohs introduced a 'radial streak disk method' with 15mm diameter disks. This was the first description of the comparison of a test organism with a sensitive control on the same plate. This method was the forerunner of the Stokes' technique, which until recently was still used in many laboratories in the UK. More paper disk diffusion methods were described in the mid-1940s.

Fleming's second contribution to modern antimicrobial susceptibility testing was the development of a broth dilution technique using turbidity as an end-point determination, which was the forerunner of contemporary MIC methodology. Schmith and Reymann were the first to describe an agar dilution antimicrobial susceptibility testing method [47].

At an early stage it became apparent to workers using diffusion and dilution techniques that there were many variables affecting antimicrobial susceptibility testing methods. By the end of the 1950s it was apparent that there was a need to standardize antimicrobial susceptibility testing. Several organizations and investigators began addressing this critical issue.

In 1966, significant progress in standardization of the disk method occurred when Bauer, Kirby and co-workers published their attempt to establish the disk diffusion technique as a practical method of testing with a broad application to clinical laboratories. In 1975, this method became the basis of the NCCLS disk diffusion standards [47].

The primary function of in-vitro antimicrobial susceptibility testing in clinical laboratories is to provide information to prescribers on the choice of appropriate chemotherapy, whether it be for therapy or prophylaxis in specific patients, or to help in antimicrobial policy formulation.

Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone are not acceptable. Reliable results can only be obtained with disk diffusion tests that use the principle of standardized methodology and zone diameter measurements. The outer limit of the zone of inhibition contains an antimicrobial concentration, which is similar to the MIC of that antimicrobial to a specific organism.

The zone diameters for individual antimicrobials are reported as susceptible, intermediate, or resistant by referring to an interpretative chart.

The definition of these categories is as follows [29]:

Susceptible: A category that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species.

Intermediate: A category that implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of the drug can be used; also indicates a "buffer zone" that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

Resistant: Resistant isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or fall in the range where specific resistance mechanisms are likely (e.g., beta-lactamases), and clinical efficacy has not been reliable in treatment studies.

In order to categorize strains as susceptible, intermediate or resistant, breakpoint antibiotic concentrations are used. A breakpoint is a discriminating MIC value used to define isolates as susceptible, intermediate or resistant.

Three features of both the antimicrobial agent and the pathogen must be considered when deciding upon a breakpoint: **(i)** the bacterial population distribution of MIC values; **(ii)** pharmacological properties of the antimicrobial; and **(iii)** clinical outcome data [26].

The rationale of the interpretative charts assumes that the relationship between the diameter of the zone and the MICs is known. In order to define this relationship, scattergram plots are generated for each of the antimicrobial/organism combinations studied.

The MIC values plotted against matching zone diameters result in a 'scattergram', from which the line of best fit (regression line) is calculated. Zone diameters, equivalent to the breakpoints, to separate the different categories, susceptible, intermediate or resistant are determined from this linear relationship [5, 29].

All the susceptibility-testing techniques described above require the prior isolation and identification of the organism, and hence the result is often not available until 2 days later. There is also no international agreement on breakpoints for interpretation of antimicrobial susceptibility tests.

To increase the speed and reliability of resistance testing, the use of a genotypic approach has been advocated recently [7], and numerous DNA-based assays have been developed for detection of bacterial resistance genes.

Bergeron and Ouellette [7] highlighted the limitations of this approach. For example the presence of a resistance gene may not always be indicative of resistant bacteria, and conversely, if a gene coding for resistance to an antimicrobial is not detected, it may not mean that the bacteria are susceptible to that particular agent. Further clinical studies will be required to validate the genotypic approach to testing for resistance,

THE STUDY:

Materials and methods

Bacterial strains:

One hundred and seventy two clinical isolates of *Acinetobacter* spp. from the Groote Schuur Hospital region, Cape Town, were collected both retrospectively (Feb 1998 to Dec 2000) and during the evaluation period (Jan to Jun 2001).

Only one isolate per patient was included; however organisms isolated from the same patient on more than one occasion were included if these repeat isolates showed different antimicrobial susceptibility patterns. This approach is based on a study [11], which showed that the use of antimicrobial susceptibility patterns to group similar *Acinetobacter* isolates correlates well with other typing and epidemiological data.

Bacterial identifications:

Phenotypic identification of the genus *Acinetobacter* was based on the following criteria [18]: gram-negative coccoid rods, oxidase negative, catalase positive, non-fermenting and strict aerobes.

Phenotypic identification of Acinetobacters to the species level was performed utilising simplified phenotypic tests (Table 1), based on tests described by Gerner-Smidt *et al* and others [16, 34, 39].

Tests for growth at 35°C and 44°C were performed using Mueller-Hinton agar (Biolab.Merk) utilising an incubator. Sheep blood (5%) agar plates were used to detect haemolysis, glucose oxidation was determined in a 1% dextrose medium, and citrate utilisation was determined using Simmons citrate medium (Oxoid). The above tests were incubated at 35°C, and results read after 18 to 24 hours and again after 48 hours to confirm the negative results.

Table 1. Phenotypic characteristics of *Acinetobacter* spp.

	A.calcoaceticus	*A.baumanii*	*A.haemolyticus*	*A.junii*	*A.lwoffii*
Growth at 37°C	100	100	100	100	100
Growth at 44°C	0	100	0	0	0
Acid from glucose	100	95	52	0	6
Sheep blood agar (haemolysis)	0	0	100	0	0
Citrate utilisation	100	100	91	82	0

* The numbers represent the % positive strains for each test.

♦ Data are from the Manual of Clinical Microbiology, 7th edition, p-542 [39].

A summary of how the species of Acinetobacter were identified:

- Glucose oxidizing, non-haemolytic strains with growth at 44°C were identified as *A.baumanii*.
- Glucose oxidizing, non-haemolytic strains with no growth at 44°C were identified as *A.calcoaceticus*.
- Glucose negative, non-haemolytic and citrate positive were identified as *A.junii*.
- Glucose negative, non-haemolytic and citrate negative were identified as *A.lwoffii*.
- Haemolytic strains were identified as *A.haemolyticus*

NB. *A.johnsonii* will not be identified by this scheme, because of its inability to grow at 35°C or more.

After the bacterial identifications were confirmed, the isolates were stored on Dorset egg media at room temperature. At a later stage the antimicrobial susceptibilities were tested in batches.

Antimicrobial agents:

For disk diffusion tests CPIM (30μg), CTAZ (30μg) and TMP-SULFA (1.25 μg/23.75μg) were obtained from Oxoid, and PIP-TAZO (100μg/10μg) was obtained from Mast diagnostics. For MIC determinations CPIM and CTAZ E-strips were obtained from AB Biodisk Slona, Sweden, TMP-SULFA reference powders were obtained from Mast diagnostics, and PIP-TAZO reference powders were obtained from Wyeth-Ayerst.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing was performed as recommended by the NCCLS [28, 30].

The disk diffusion test was performed, using MH agar (Biolab Merk), to determine the zone size diameters for CPIM, CTAZ, TMP-SULFA and PIP-TAZO. The MIC was determined by both the agar dilution method for TMP-SULFA and PIP-TAZO, and the E-test for CPIM and CTAZ, using MH agar (Biolab Merk) in both methods.

[a] Disk diffusion tests:

Inoculum preparation:

A direct broth suspension of isolated colonies selected from a 16 to 18 hours growth on an agar plate was performed, using Mueller-Hinton broth. The suspension was then adjusted to match a 0.5 McFarland turbidity standard by the unaided eye.

Inoculation of test plates:

A sterile cotton swab was dipped into the adjusted suspension, rotated and pressed to the inside of the test tube to remove excess fluid from the swab. The dried surface of a Mueller-Hinton agar plate was then inoculated with the swab using a rotating device.

Application of the antimicrobial disks:

The antimicrobial disks (viz. CPIM, CTAZ, TMP-SULFA, and PIP-TAZO) were dispensed onto the inoculated agar using a dispensing apparatus, making sure that they are no closer than 24mm from centre to centre. Plates were inverted and placed in an incubator set at 35°C.

Reading plates:

After 16 to 18 hours each plate was examined to check that the inoculum was correct (i.e. semiconfluent). The zones of inhibition were measured to the nearest millimetre, using sliding calipers. For TMP-SULFA slight growth (20% or less of the lawn of growth) was disregarded, and the more obvious margin was measured.

The zone diameters were recorded in millimetres for each antimicrobial agent tested against each isolate.

[b] Agar dilution method:

The agar dilution method was used to determine the MIC for TMP-SULFA and PIP-TAZO.

Our intention was to also use this method to determine the MIC for CPIM and CTAZ, but the reference powders of CPIM and CTAZ were not obtainable, hence it was decided to perform the E-test to determine their MICs.

Weighing antimicrobial powders:

The following formula [30] was used to determine the amount of reference powder needed for the preparation of the antimicrobial stock solution:

$$\text{Weight (mg)} = \frac{\text{Volume (ml) x Concentration (µg/ml)}}{\text{Assay potency (µg/ml)}}$$

Preparation of stock solutions:

The procedure for TMP-SULFA and PIP-TAZO stock solution preparation, from the reference powder, is shown in Table 2.

Table 2. TMP-SULFA and PIP-TAZO stock solutions preparation.

Antimicrobial powder	**Solvent**	**Diluents**	**Concentration of stock solutions (µg/ml)**	**Dilution range tested (µg/ml)**
Trimethoprim	0.05 mol/L hydrochloric acid (10% final volume).	Water heated to 35°C in a water bath.	320	0.016 to 32
Sulfamethoxazole	Half volume hot water and minimum amount of 2.5 mol/L NaOH to dissolve.	Water	6080	0.29 to 608
Piperacillin - sodium	Water	Water	5120	0.125 to 512
Tazobactam	Water	Water	40	4 (Constant throughout).

NB. Piperacillin monohydrate powder supplied by Wyeth-Ayerst will only dissolve in pure methanol, and as soon as water (hot or cold) is added it will precipitate. Wyeth-Ayerst has recommended the use of piperacillin-sodium powder, which is soluble in water, for MIC determination by the agar dilution method.

The stock solutions were dispensed in aliquots into sterile polystyrene test tubes, and then stored at -20°C, until used.

Preparing agar dilution plates:

TMP-SULFA was tested at a ratio of 1/19 (0.016/0.29μg/ml to 32/608μg/ml) and PIP-TAZO was tested at 0.125/4μg/ml to 512/4μg/ml. Tazobactam concentration was constant at 4μg/ml throughout the dilutions of piperacillin.

Mueller-Hinton agar (MH-agar) was used for the agar dilution method, with a final volume of 20ml per 9cm petri dish plates to give an agar depth of approximately 4mm.

The appropriate dilutions of the antimicrobial agent were added to molten Mueller-Hinton agar that had been allowed to equilibrate in a water bath set at 50°C.

The agar and antimicrobial dilution were mixed thoroughly before being poured into 9cm petri dish plates. The agar was allowed to cool and solidify at room temperature, making sure no bubbles were formed in this process.

The plates were then sealed in plastic bags and stored at 4–8°C for no longer than 5 days.

Growth control plates:

Antimicrobial free Mueller-Hinton agar plates were prepared for use as growth control plates.

Preparing the inoculum:

Bacterial suspensions adjusted to match a 0.5 McFarland standard were prepared as described earlier for the disk susceptibility technique. These adjusted suspensions contain approximately 1 to 2 X 10^8 CFU/ml. This was further diluted 1:10 in sterile normal saline to obtain the desired inoculum concentration of 10^7 CFU/ml.

Inoculating the agar dilution plates:

The agar plates were inoculated with a semi-automated replicating device.

An aliquot (100µl) of the adjusted and diluted bacterial suspension was transferred to the corresponding well in the replicator inoculum block. The agar plates were marked for orientation.

Approximately 1 to 2µl was transferred from each well in the inoculum block onto the agar surface by the replicating device.

A growth control plate was inoculated first and then the agar dilution plates were inoculated starting with the lowest antimicrobial concentration. A second growth control plate was included at the end to ensure that there was no contamination or significant antimicrobial carryover during the inoculating process. The first growth control plate was used to check that the organisms were viable and could grow on Mueller-Hinton agar plates.

The inoculated plates were left at room temperature till the inoculum spots were absorbed into the agar, and then they were inverted and incubated at 35° C for 18 to 24 hours.

Determining agar dilution end points:

The plates were read against a dark background, with an incident light source. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibited growth. A single colony or a faint haze was disregarded.

[c] The E-test method:

The E-test method (E-test AB Biodisk, Slona, Sweden) was used for MIC determination of cefepime and ceftazidime.

Cefepime E-test strips have an antimicrobial concentration range of 0.002 to 32 μg/ml, and ceftazidime E-test strips have an antimicrobial concentration range of 0.016 to 256 μg/ml.

The MIC determination was performed as recommended by the manufacturer.

Inoculum preparation:

Bacterial suspensions adjusted to match a 0.5 McFarland standard were prepared as described earlier for the disk susceptibility technique.

Inoculation:

Mueller-Hinton agar was used based on the NCCLS recommendations. A sterile swab was dipped into the inoculum suspension, and excess fluid removed by pressing the swab against the inside wall of the test tube. Then the entire surface of a 9cm petri dish plate was swabbed three times, rotating the plate approximately 90 degrees each time, to ensure an even distribution of the inoculum.

Application of the E-test strips:

The E-test strip was applied manually to a dry inoculated MH-agar, making sure that the whole length of the strip was in complete contact with the agar surface. If any, air pockets were removed by pressing gently on the strip with forceps, moving from the minimum concentration upwards.

Two E-test strips were applied to a single 9cm agar plate, in parallel, but with the gradient antimicrobial concentrations running in opposite directions.

Incubation:

Agar plates were incubated immediately at 35°C for 18 to 20 hours.

Reading the MIC:

The MIC was determined at the point of intersection between the inhibition ellipse edge and the E-test strip. When growth occurred along the strip, i.e. no inhibition ellipse seen, the MIC was reported as "greater than" (>32μg/ml for CPIM and >256μg/ml for CTAZ). When the inhibition ellipse is below the strip, i.e. the zone edge does not intercept the strip; the MIC was reported as "less than" (<0.002μg/ml for CPIM and <0.016μg/ml for CTAZ).

NB. The MIC was always read at the point of complete inhibition of all growth. Hazy growth and isolated colonies were regarded as growth.

Quality control procedures:

[a] For the disk diffusion tests the following, reference strains were included in each run:

Escherichia coli	**ATCC 25922**
Escherichia coli	**ATCC 35218**
Pseudomonas aeruginosa	**ATCC 27853**

[b] For the agar dilution method:

For TMP-SULFA, the following reference strain was included in each run:

Escherichia coli	**ATCC 25922**

For PIP-TAZO, the following reference strains were included in each run:

Escherichia coli	**ATCC 35218**

[c] For the E-tests for CPIM and CTAZ, the following reference strains were included in each run:

Escherichia coli	**ATCC 25922**
Pseudomonas aeruginosa	**ATCC 27853**

The Acinetobacter isolates were tested in batches of 18 organisms per run. Zone size diameters and MICs were determined simultaneously, and quality control reference strains were included with each run.

The results of each run were only accepted when the quality control reference strains results were within the acceptable ranges recommended by NCCLS (shown in Tables 4 and 5 in appendix). [31].

Regression line determination:

The MIC values, expressed logarithmically, were plotted against matching zone diameters resulting in a "scattergram". The regression line was calculated, correlating MICs and zone diameters of inhibition. These calculations excluded undefined measurements (such as no zone of inhibition or off-scale MICs) [29].

The Pearson coefficient of correlation was used as a measure of the linear relationship obtained between the MIC and zone diameters of inhibition for each antimicrobial agent tested [23].

The use of a regression line allows the determination of the equivalent zone size diameter for each MIC, however interpretative zone size diameters can only be established when the MIC breakpoints have been selected [1]. The MIC breakpoints used in the study were those selected by the NCCLS for *Acinetobacter* spp. [31].

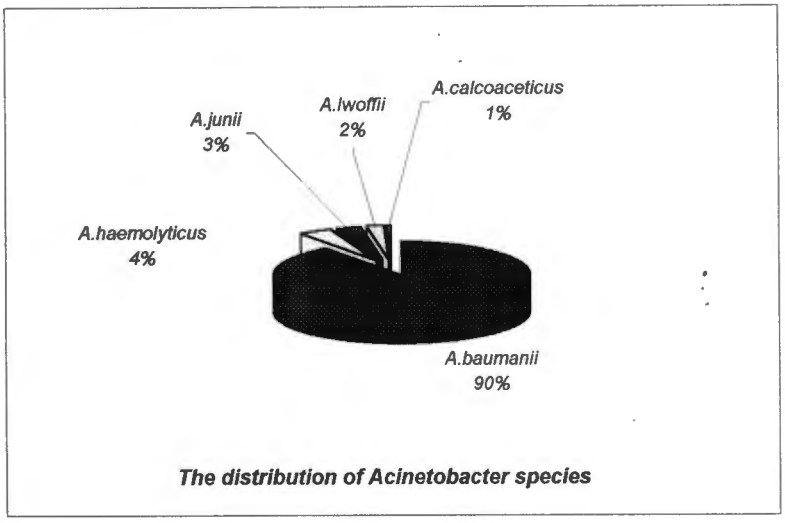
REULTS:

Bacterial strains tested:

One hundred and seventy two Acinetobacter isolates were included in this study, of which 160 were tested for their antimicrobial susceptibilities to CPIM, CTAZ, TMP-SULFA and PIP-TAZO. The distributions of species were as follows (Figure 1): *A.baumanii* (155 [90%]), *A.haemolyticus* (7 [4.1%]), *A.junii* (5 [3%]), *A.lwoffii* (4 [2.3%]) and *A.calcoaceticus* (1 [0.6%]).

The Acinetobacter isolates included in this study (both those collected retrospectively and during the study period) were mostly isolated from blood culture and tracheal aspirate specimens from ICU patients, and they do not reflect the overall incidence and prevalence of *Acinetobacter* spp. in the Groote Schuur Hospital region.

Figure 1.



The distribution of Acinetobacter species

Phenotypic identification and antimicrobial susceptibility testing results:

Table 3. (see appendix), shows the phenotypic identification and susceptibility testing results for the individual Acinetobacter isolates.

Controls:

The results of the control reference strains were within the acceptable ranges recommended by NCCLS [31] in all 9 testing runs. There were no runs rejected and repeated. Table 6. (see appendix), shows the results obtained for each control reference strain in each testing run.

For the acceptable ranges recommended by the NCCLS for these control reference strains see Tables 4 and 5 (in appendix).

Antimicrobial susceptibility interpretation:

The antimicrobial susceptibility testing results (zone diameters and MICs) were interpreted as sensitive, intermediate and resistant by referring to the NCCLS interpretative standards for *Acinetobacter* spp. (Table 7.) [31].

Table 7. The NCCLS zone diameter interpretative standards and the equivalent MIC breakpoints for *Acinetobacter* spp.

Antimicrobial agent	MIC breakpoint (μg/ml)		Disk content (μg)	Interpretative zone diameter (mm)		
	R	S		R	I	S
CTAZ	≥32	≤8	30	≤14	15-17	≥18
CPIM	≥32	≤8	30	≤14	15-17	≥18
TMP-SULFA	≥8/152	≤2/38	1.25/23.75	≤10	11-15	≥16
PIP-TAZO	≥128/4	≤16/4	100/10	≤17	18-20	≥21

R= Resistant, I= Intermediate, and S= Sensitive.

Agreements and errors:

Disk diffusion and MIC results were deemed to be in agreement if the test results were within the same susceptibility category, and the errors were defined as in the NCCLS M23-A2 publication [29].

The errors were ranked as follows: **very major error**, false susceptible result produced by the disk diffusion test; **major error**, false resistant result produced by the disk diffusion test; and **minor error**, when one test result is intermediate and the other is susceptible or resistant.

Table 8. summarizes all the interpretative errors of the disk diffusion test results, (interpreted using the NCCLS zone diameter interpretative standards), compared to the MIC result, for each antimicrobial agent tested.

Table 8. Summary of the interpretative errors for the tested antimicrobial agents.

Antimicrobial agents tested	**Interpretative errors**		
	Very major	**Major**	**Minor**
CPIM	13 (8%)	0	63 (39.4%)
CTAZ	0	0	20 (12.5%)
TMP-SULFA	0	4 (2.5%)	23 (14.4%)
PIP-TAZO	3 (1.9%)	1 (0.63%)	49 (31%)

The disk susceptibility for CTAZ and TMP-SULFA showed no very major errors. In addition CTAZ showed no major errors either, but TMP-SULFA showed 4 major errors. CPIM showed 13 (8%) very major errors and no major errors, and PIP-TAZO showed 3 (1.9%) very major errors and one major error. Minor interpretative errors were frequent with all the antimicrobial agents tested.

Regression line determination:

Figures 2 to 5 (see appendix) present the regression lines calculated for CPIM, CTAZ, TMP-SULFA, and PIP-TAZO respectively. The NCCLS breakpoints and zone size criteria, as well as the zone size diameters determined in the study, are indicated on each figure.

The regression line for each antimicrobial/organism was calculated excluding the undefined measurements (such as no zone of inhibition or off-scale MIC), (Table 9).

Table 9. The number of Acinetobacter isolates included in the regression line calculation for each antimicrobial agent tested.

Antimicrobial agent tested	Number of Acinetobacter isolates included for regression line calculation.
CPIM	87
CTAZ	159
TMP-SULFA	68
PIP-TAZO	118

The Pearson coefficient of correlation calculated for each antimicrobial agent is as follows:

CPIM = - 0.72868
CTAZ = - 0.70981
TMP-SULFA = - 0.76087
PIP-TAZO = - 0.40866

For CPIM, CTAZ and TMP-SULFA there is a strong negative relationship between the MICs and zone size diameters.

For PIP-TAZO the relationship is moderate.

DISCUSSION:

The aim of the study is to evaluate the appropriateness/validity of the NCCLS zone size criteria for *Acinetobacter* spp. in an effort to enhance the reliability of in-vitro antimicrobial susceptibility results for *Acinetobacter* spp.

To our knowledge there were no published reports on the validity of the NCCLS breakpoints for *Acinetobacter* spp.

An increased number of nosocomial *Acinetobacter* spp. are found to be resistant to multiple antimicrobial agents [8, 9, 13, 25, 41], which may indicate that there is a significant change in their population distribution [1].

Breakpoints are determined based on formulae that take into account several pharmacokinetic factors. The final adjustments to the selected breakpoint, are influenced by the bacterial population distribution, according to their susceptibility patterns to the antimicrobial agent studied.

Once the breakpoints are determined, dilution and disk diffusion tests are performed on at least 500 isolates. The data is then displayed as a scattergram with zone diameters on one axis and the MICs on another. From this scattergram the regression line is calculated representing the relationship between the zone sizes and the MICs. This relationship allows the equivalent zone size to a selected MIC breakpoint to be derived [1, 5, 29].

In practice, the proposed breakpoints are simply adjusted until the number of false sensitive disk diffusion test results (very major discrepancies) and false resistant disk tests (major discrepancies) are held to a minimum.
Minor discrepancies are also considered in these determinations [29].

In the event of a change in the bacterial population distribution, when less susceptible and/or resistant strains develop to an antimicrobial agent whose breakpoints were determined when only susceptible strains were available, the interpretative criteria may need reassessment.

When evaluating disk susceptibility tests, based on NCCLS guidelines [29], the discrepancy rates are considered acceptable if very major discrepancy rates do not exceed 1.5%, and major discrepancies do not exceed 3%, when calculated based on all isolates. These acceptable discrepancy rates are recommended for unselected clinical isolates, as is the case in this study.

This study has shown that CPIM and PIP-TAZO disk susceptibility testing has resulted in unacceptably high numbers (8% and 1.9% respectively) of very major errors (i.e. exceeding 1.5%).

None of the tested antimicrobial agents has resulted in unacceptable numbers of major errors (i.e. exceeding 3%).

It was noted that all the "very major" and "major" errors occurred when testing *A.baumanii*. This could be due to the fact that 90% of the *Acinetobacter* spp. tested in this study belonged to the species *A.baumanii*.

Minor errors were frequent with all the tested antimicrobial agents.

Regression line analysis:

For figures 2 to 5 see appendix.

[i] For CPIM, Figure 2.

The results obtained for CPIM show that the NCCLS zone size criteria are consistently smaller than those determined in this study, leading to increased false sensitive reporting. This explains the unacceptable rate of very major errors reported for CPIM disk susceptibility testing in the study.

[ii] For CTAZ, Figure 3.

The results of this study were in agreement with the NCCLS zone size criteria.

[iii] For TMP-SULFA, Figure 4.

The results obtained for TMP-SULFA show that the NCCLS zone size criteria are consistently larger than those determined in this study. This does not explain the reason for 4 (2.5%) major errors (false resistant result by disk diffusion testing) reported in the study. Three of these isolates with major errors had no zone of inhibition around the TMP-SULFA disks, but they were sensitive by the MIC testing method. This discrepancy between disk diffusion and MIC results could be related to a particular lot of Mueller-Hinton agar [42]. This warrants further investigation.

[iv] For PIP-TAZO, Figure 5.

The results of this study were in agreement with the NCCLS zone size criteria.

The unacceptable rate of very major errors reported for PIP-TAZO disk diffusion susceptibility testing in the study, may indicate a change in PIP-TAZO susceptibility pattern in this bacterial population. Such a change may necessitate a reassessment of the selected breakpoints for PIP-TAZO.

The number of Acinetobacter isolates (160) tested in this study was not sufficient to study the bacterial population distribution. Furthermore the study suffered from a further limitation. Calculation of the regression line for each antimicrobial/organism excluded the undefined measurements (such as no zone of inhibition or off-scale MIC results), resulting in further isolates being excluded, e.g. for TMP-SULFA only 68 isolates out of 160 tested were included for the regression line calculation.

Despite these limitations, this study reveals that a reassessment of the current NCCLS breakpoints and zone size criteria for *Acinetobacter* spp. may be necessary for cefepime and piperacillin/tazobactam in order to continue providing reliable in-vitro antimicrobial susceptibility testing.

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APPENDIX

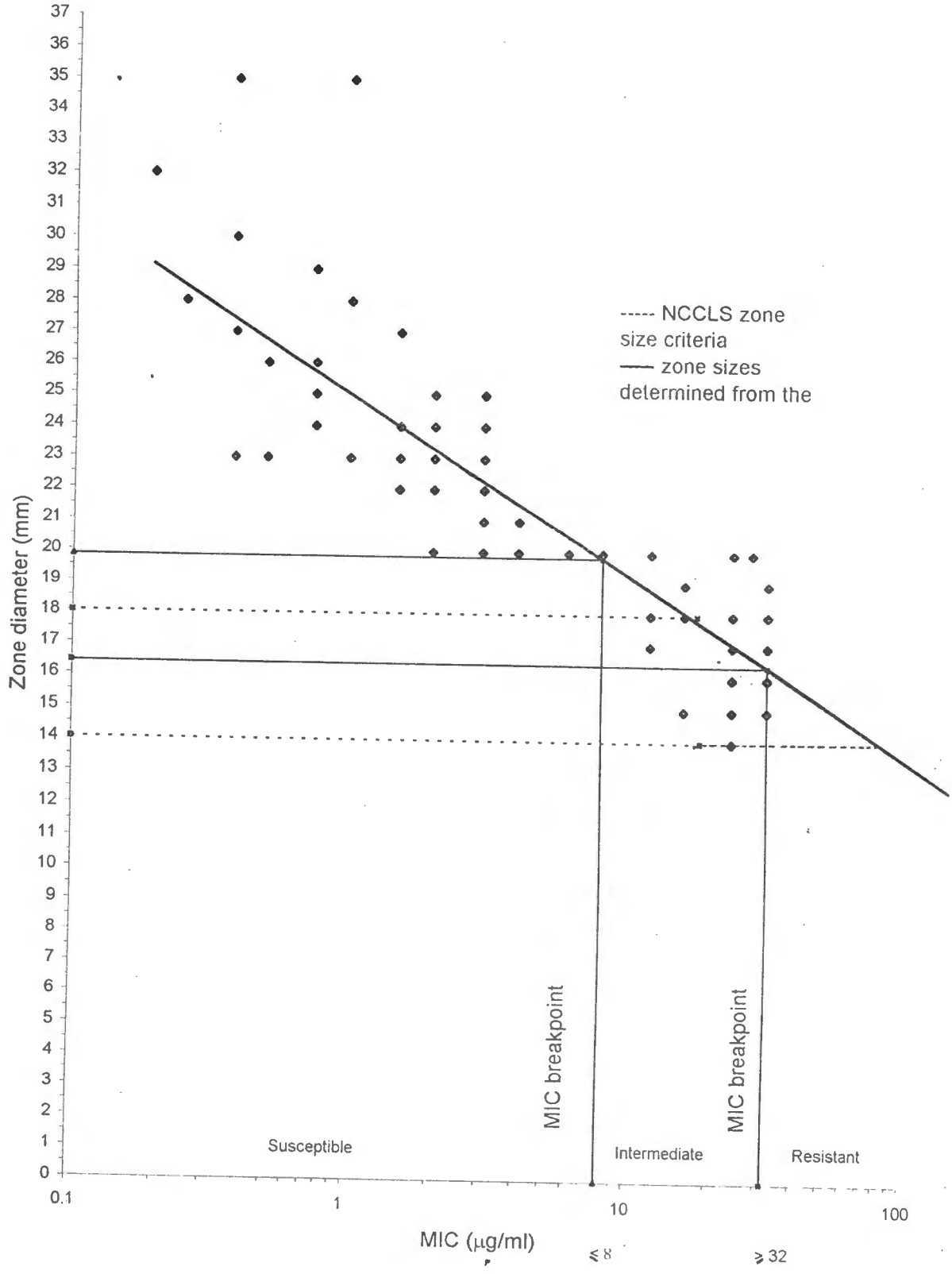

Figure 2. Regression line correlating MICs and zone diameters for cefepime. (87 *Acinetobacter* isolates).

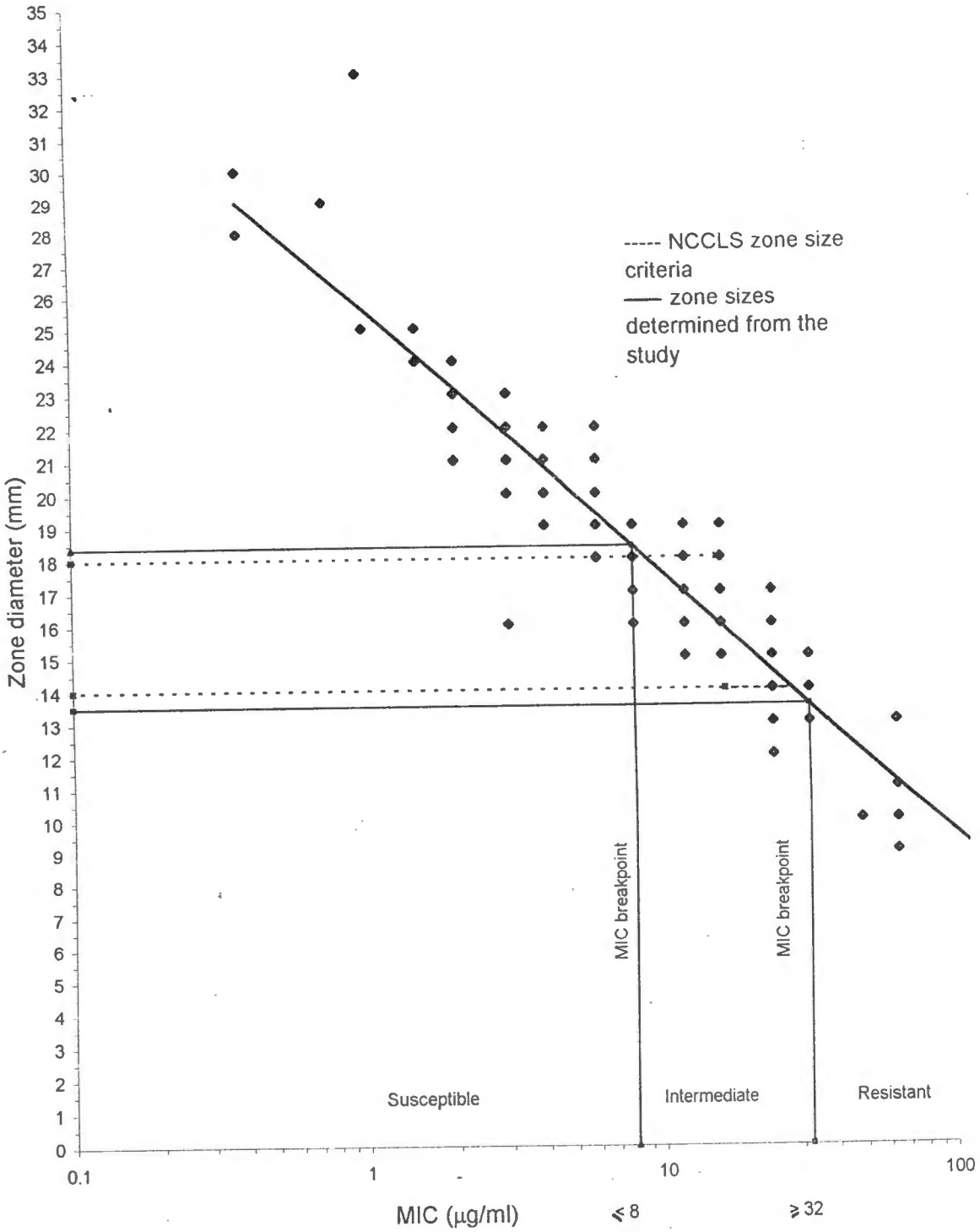

Figure 3. Regression line correlating MICs and zone diameters for ceftazidime (159 *Asinetobacter* isolates).

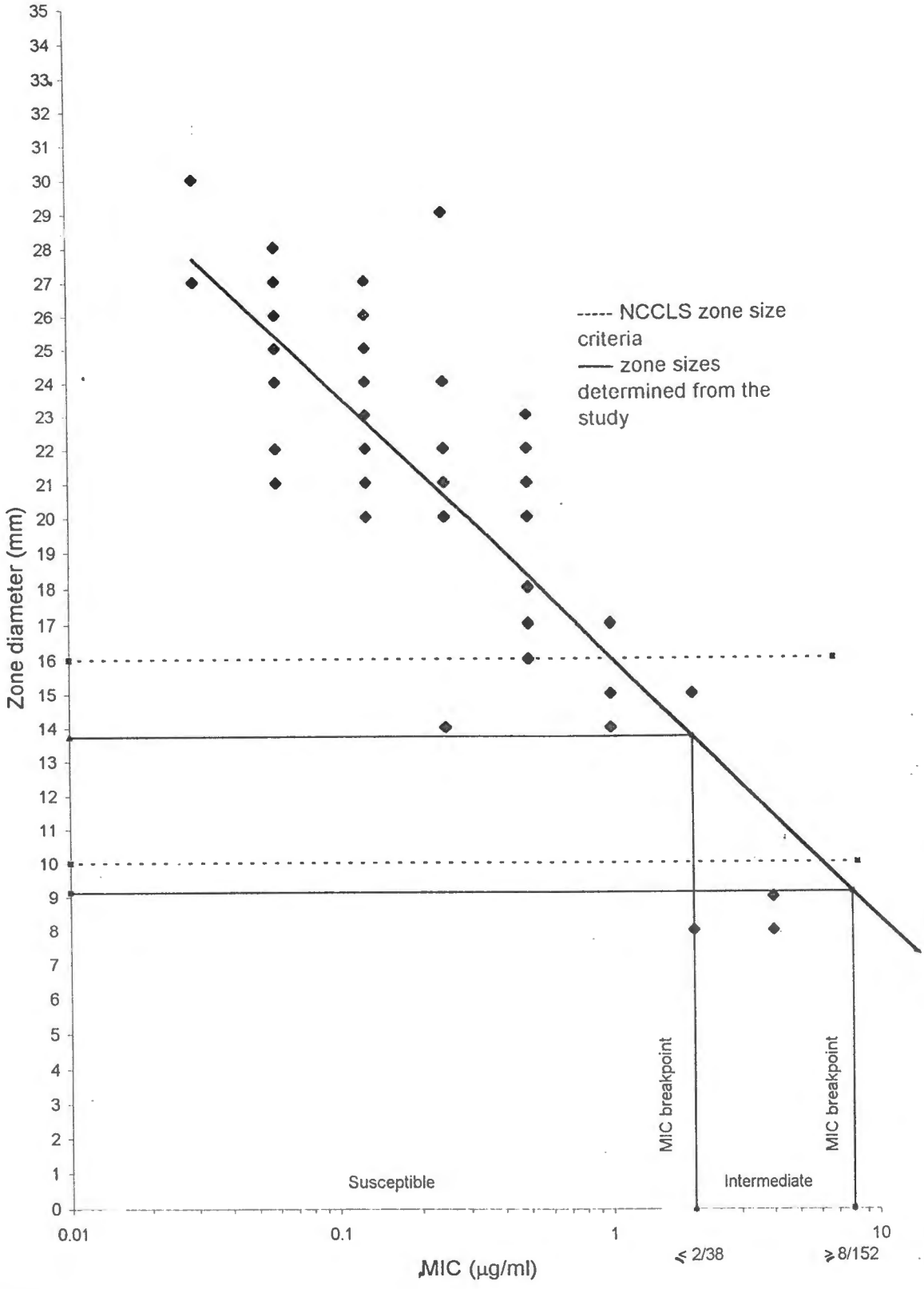

Figure 4. Regression line correlating MICs and zone diameters for trimethoprim-sulfamethoxazole (68 *Acinetobacter* isolates).

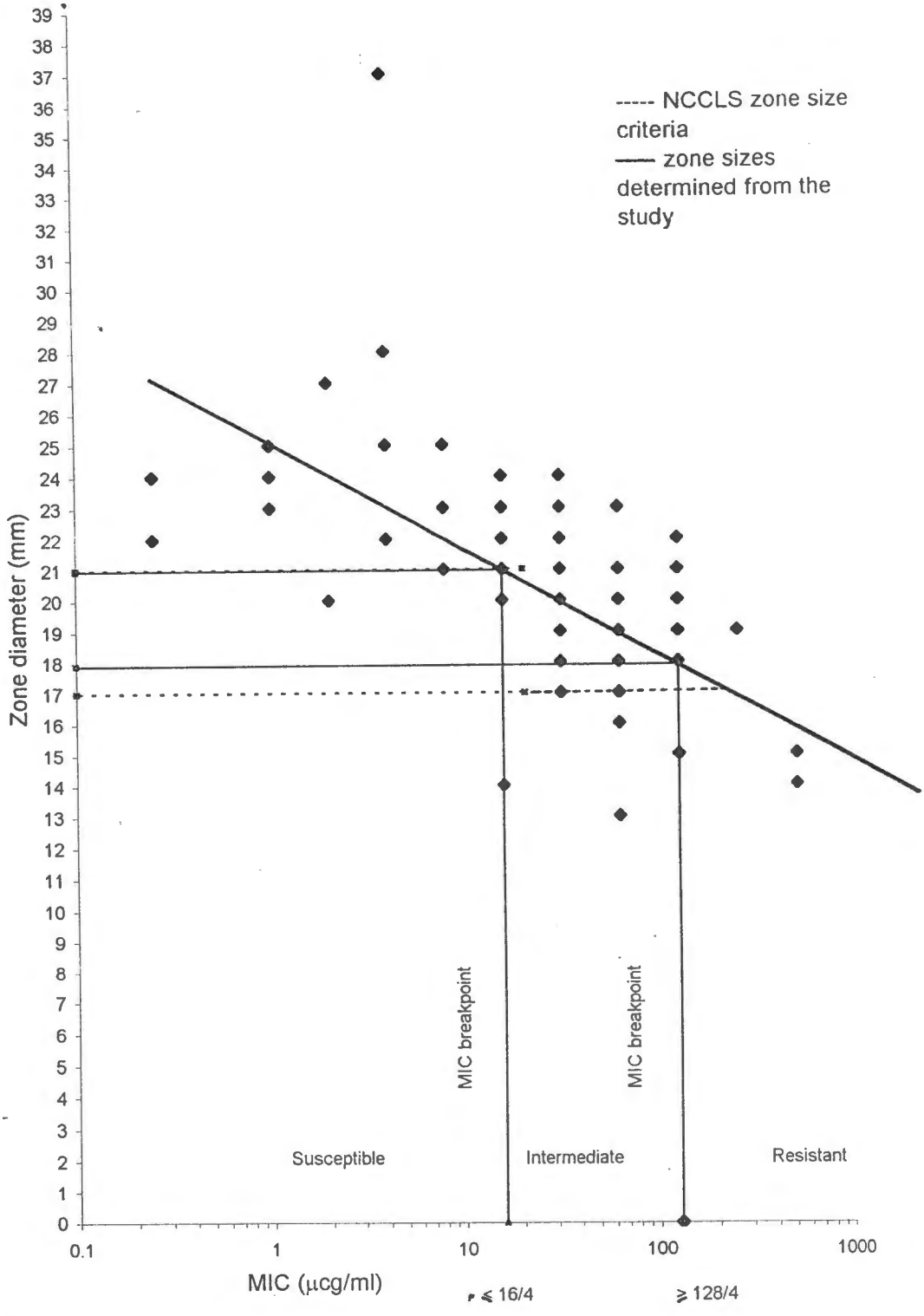

Figure 5. Regression line correlating MICs and zone diameters for piperacillin-tazobactam (118 *Acinetobacter* isolates).

Table 3 The phenotypic identification and susceptibility testing results for the individual Acinetobacter isolates.

Serial number	Phenotypic identification tests					Organism I.D.	Disk diffusion test results (mm)				E-test MIC results (μg/ml)		Agar dilution MIC results (μg/ml)	
	Acid from glucose	Citrate utilisation	Growth at 35°C	Growth at 44°C	Haemolysis on sheep blood agar		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
1	+	+	+	+	-	*A.baumanii*	19	20	0	23	16	6	>32/608	8/4
2	+	+	+	+	-	*A.baumanii*	13	19	20	26	>32	12	0.25/4.75	<0.125/4
3	+	+	+	+	-	*A.baumanii*	Non-viable							
4	+	+	+	+	-	*A.baumanii*	17	17	9	21	>32	24	4/76	64/4
5	+	+	+	NG	-	*A.calcoaceticus*	24	23	14	27	2	3	1/19	<0.125/4
6	+	+	+	+	-	*A.baumanii*	18	17	20	18	>32	12	0.5/9.5	64/4
7	+	+	+	+	-	*A.baumanii*	17	17	0	20	>32	16	>32/608	16/4
8	+	+	+	+	-	*A.baumanii*	18	19	0	18	32	12	>32/608	32/4
9	+	+	+	+	-	*A.baumanii*	16	17	0	17	>>32	16	32/608	64/4
10	+	+	+	+	-	*A.baumanii*	17	16	0	21	>32	24	4/76	64/4
11	+	+	+	+	-	*A.baumanii*	35	33	14	45	0.38	1.0	0.25/4.75	<0.125/4
12	+	+	+	+	-	*A.baumanii*	13	13	8	20	>32	64	2/38	64/4
13	+	+	+	+	-	*A.baumanii*	18	19	21	19	>32	12	0.25/4.75	64/4
14	+	+	+	+	-	*A.baumanii*	23	21	25	25	2	3	0.06/1.19	<0.125/4
15	-	+	+	+	-	*A.baumanii*	35	16	0	37	1	3	>32/608	4/4
16	+	+	+	+	-	*A.baumanii*	20	19	0	22	24	16	4/76	16/4
17	+	+	+	+	-	*A.baumanii*	26	25	24	27	0.75	1.5	0.13/2.38	<0.125/4
18	+	+	+	+	-	*A.baumanii*	17	17	0	17	>32	12	>32/608	64/4
19	+	+	+	+	-	*A.baumanii*	16	14	15	22	>32	24	2/38	128/4
20	+	+	+	+	-	*A.baumanii*	21	19	18	28	>32	12	0.5/9.5	<0.125/4
21	+	+	+	+	-	*A.baumanii*	16	15	17	23	>32	16	1/19	64/4
22	-	+	+	+	-	*A.baumanii*	14	20	22	28	>32	6	0.13/2.38	<0.125/4
23	+	+	+	-	+	*A.haemolyticus*	Non-viable							
24	+	+	+	+	-	*A.baumanii*	Non-viable							
25	+	+	+	+	-	*A.baumanii*	Non-viable							
26	+	+	+	+	-	*A.baumanii*	28	25	0	28	1.0	1.5	16/304	<0.125/4
27	+	+	+	+	-	*A.baumanii*	18	18	0	20	>32	8	32/608	64/4
28	+	+	+	+	-	*A.baumanii*	20	20	0	25	28	6	>32/608	8/4

Serial number	Phenotypic identification tests					Organism I.D.	Disk diffusion test results (mm)				E-test MIC results (μg/ml)		Agar dilution MIC results (μg/ml)	
	Acid from glucose	Citrate utilisation	Growth at 35°C	Growth at 44°C	Haemolysis on sheep blood agar		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
29	+	+	+	+	-	*A.baumanii*	17	17	0	19	>32	8	32/608	128/4
30	+	+	+	+	-	*A.baumanii*	17	16	0	20	>32	16	4/76	64/4
31	+	+	+	+	-	*A.baumanii*	18	18	0	16	32	8	32/608	64/4
32	+	+	+	+	-	*A.baumanii*	17	16	0	20	>32	12	4/76	64/4
33	+	+	+	+	-	*A.baumanii*	16	16	0	17	>32	16	16/304	64/4
34	+	+	+	+	-	*A.baumanii*	25	23	23	27	3	3	0.13/2.38	2/4
35	+	+	+	+	-	*A.baumanii*	13	19	22	27	>32	8	0.13/2.38	<0.125/4
36	+	+	+	+	-	*A.baumanii*	19	19	0	19	16	6	>32/608	32/4
37	+	+	+	+	-	*A.baumanii*	29	25	0	27	0.75	1.5	8/152	<0.125/4
38	+	+	+	+	-	*A.baumanii*	Non-viable							
39	+	-	+	-	+	*A.haemolyticus*	32	28	25	35	0.19	0.38	0.13/2.38	<0.125/4
40	+	+	+	+	-	*A.baumanii*	16	16	0	19	>32	12	32/608	64/4
41	+	+	+	+	-	*A.baumanii*	17	16	0	21	>32	16	4/76	64/4
42	+	+	+	+	-	*A.baumanii*	23	20	20	22	2	3	0.13/2.38	4/4
43	+	+	+	+	-	*A.baumanii*	17	16	0	15	32	16	>32/608	128/4
44	+	+	+	+	-	*A.baumanii*	17	15	16	21	>32	16	0.5/9.5	64/4
45	+	+	+	+	-	*A.baumanii*	16	16	0	17	32	12	16/304	64/4
46	+	+	+	+	-	*A.baumanii*	17	16	0	20	32	12	2/38	32/4
47	+	+	+	+	-	*A.baumanii*	16	10	0	13	>32	48	32/608	64/4
48	+	+	+	+	-	*A.baumanii*	20	18	0	18	6	6	>32/608	128/4
49	+	+	+	+	-	*A.baumanii*	16	19	0	23	>32	6	>32/608	8/4
50	+	+	+	+	-	*A.baumanii*	19	17	0	23	32	12	>32/608	16/4
51	+	+	+	+	-	*A.baumanii*	18	16	0	20	>32	12	2/38	32/4
52	+	+	+	+	-	*A.baumanii*	23	22	21	25	1.5	2	0.06/1.19	1/4
53	+	+	+	+	-	*A.baumanii*	8	15	0	21	>32	24	4/76	8/4
54	+	+	+	+	-	*A.baumanii*	16	16	0	20	>32	16	2/38	32/4
55	+	+	+	+	-	*A.baumanii*	14	17	0	20	>32	12	>32/608	128/4
56	+	+	+	+	-	*A.baumanii*	19	19	0	23	32	6	>32/608	32/4
57	+	+	+	+	-	*A.baumanii*	Non-viable							

Serial number	Phenotypic identification tests					Organism I.D.	Disk diffusion test results (mm)				E-test MIC results (μg/ml)		Agar dilution MIC results (μg/ml)	
	Acid from glucose	Citrate utilisation	Growth at 35°C	Growth at 44°C	Haemolysis on sheep blood agar		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
58	+	+	+	+	-	*A.baumanii*	16	17	0	21	>32	16	>32/608	8/4
59	+	+	+	+	-	*A.baumanii*	23	21	20	24	2	3	0.13/2.38	1/4
60	+	+	+	+	-	*A.baumanii*	16	16	0	18	>32	16	16/304	64/4
61	+	+	+	+	-	*A.baumanii*	19	21	0	18	>32	6	8/152	64/4
62	+	+	+	+	-	*A.baumanii*	15	16	0	18	>32	16	>32/608	64/4
63	+	+	+	+	-	*A.baumanii*	16	16	0	17	24	8	32/608	32/4
64	+	+	+	+	-	*A.baumanii*	18	15	20	18	16	24	0.13/2.38	128/4
65	+	+	+	+	-	*A.baumanii*	15	15	0	18	32	12	8/152	64/4
66	+	+	+	+	-	*A.baumanii*	15	15	0	18	32	12	8/152	32/4
67	+	+	+	+	-	*A.baumanii*	18	15	21	20	12	24	0.13/2.38	128/4
68	+	+	+	+	-	*A.baumanii*	14	16	0	20	24	12	16/304	64/4
69	+	+	+	+	-	*A.baumanii*	Non-viable							
70	+	+	+	+	-	*A.baumanii*	18	15	21	19	16	24	0.25/4.75	128/4
71	+	+	+	+	-	*A.baumanii*	Non-viable							
72	+	+	+	+	-	*A.baumanii*	16	10	0	15	>32	64	>32/608	512/4
73	+	+	+	+	-	*A.baumanii*	14	14	0	17	>32	24	8/152	64/4
74	+	-	+	-	-	*A.lwoffii*	24	24	15	28	1.5	1.5	1/19	<0.125/4
75	+	+	+	+	-	*A.baumanii*	15	15	0	14	>32	24	8/152	16/4
76	+	+	+	+	-	*A.baumanii*	18	18	23	20	12	6	0.5/9.5	32/4
77	+	+	+	+	-	*A.baumanii*	17	17	22	19	>32	16	0.25/4.75	128/4
78	+	+	+	NG	+	*A.haemolyticus*	24	22	22	25	0.75	3	0.13/2.38	<0.125/4
79	+	+	+	+	-	*A.baumanii*	20	20	20	22	4	4	0.25/4.75	4/4
80	+	+	+	+	-	*A.baumanii*	17	17	20	19	12	8	0.5/9.5	32/4
81	+	+	+	+	-	*A.baumanii*	21	20	22	24	3	3	0.13/2.38	<0.125/4
82	-	+	+	NG	+	*A.haemolyticus*	Non-viable							
83	+	+	+	+	-	*A.baumanii*	20	20	22	22	3	4	0.13/2.38	16/4
84	+	+	+	+	-	*A.baumanii*	22	22	8	26	1.5	3	4/76	<0.125/4
85	+	+	+	+	-	*A.baumanii*	Non-viable							
86	-	+	+	NG	-	*A.junii*	23	21	27	26	1.5	4	0.03/0.59	<0.125/4

Serial number	Phenotypic identification tests					Organism I.D.	Disk diffusion test results (mm)				E-test MIC results (μg/ml)		Agar dilution MIC results (μg/ml)	
	Acid from glucose	Citrate utilisation	Growth at 35°C	Growth at 44°C	Haemolysis on sheep blood agar		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
87	+	+	+	+	-	*A.baumanii*	12	20	21	26	>32	6	0.13/2.38	<0.125/4
88	+	+	+	+	-	*A.baumanii*	Non-viable							
89	+	+	+	+	-	*A.baumanii*	20	21	22	22	4	4	0.25/4.75	16/4
90	+	+	+	+	-	*A.baumanii*	Non-viable							
91	+	+	+	+	-	*A.baumanii*	21	21	0	23	3	3	16/304	8/4
92	+	+	+	+	-	*A.baumanii*	15	18	0	17	16	8	32/608	64/4
93	+	+	+	+	-	*A.baumanii*	22	22	22	27	3	2	0.06/1.19	<0.125/4
94	+	+	+	+	-	*A.baumanii*	13	15	0	19	>32	24	>32/608	64/4
95	+	+	+	+	-	*A.baumanii*	14	14	0	20	>32	24	>32/608	64/4
96	+	+	+	+	-	*A.baumanii*	20	20	23	24	3	4	0.13/2.38	<0.125/4
97	+	+	+	+	-	*A.baumanii*	18	20	0	22	24	6	>32/608	16/4
98	+	+	+	+	-	*A.baumanii*	9	15	0	21	>32	16	>32/608	64/4
99	+	+	+	+	-	*A.baumanii*	16	18	21	19	32	8	0.5/9.5	64/4
100	+	+	+	+	-	*A.baumanii*	16	15	0	22	>32	16	4/76	32/4
101	+	+	+	+	-	*A.baumanii*	22	22	24	25	2	3	0.06/1.19	<0.125/4
102	-	+	+	NG	-	*A.junii*	27	22	30	28	1.5	6	0.03/0.59	4/4
103	-	-	+	NG	-	*A.lwoffii*	27	24	0	27	0.38	1.5	4/76	<0.125/4
104	+	+	+	+	-	*A.baumanii*	17	16	0	26	24	16	8/152	<0.125/4
105	+	+	+	+	-	*A.baumanii*	20	17	22	18	12	16	0.25/4.75	128/4
106	+	+	+	+	-	*A.baumanii*	21	22	0	21	3	3	8/152	16/4
107	+	+	+	+	-	*A.baumanii*	22	22	21	22	1.5	3	0.5/9.5	4/4
108	+	+	+	+	-	*A.baumanii*	11	12	0	15	>32	24	32/608	128/4
109	+	+	+	+	-	*A.baumanii*	14	16	0	17	>32	16	32/608	64/4
110	+	+	+	+	-	*A.baumanii*	10	20	22	22	>32	6	0.25/4.75	<0.125/4
111	-	+	+	NG	-	*A.junii*	28	30	27	30	0.25	0.38	0.13/2.38	<0.125/4
112	+	+	+	+	-	*A.baumanii*	16	18	0	18	24	8	32/608	64/4
113	-	+	+	NG	-	*A.junii*	25	21	28	27	2	6	0.06/1.19	<0.125/4
114	+	+	+	+	-	*A.baumanii*	15	16	0	17	>32	16	4/76	32/4
115	+	+	+	NG	+	*A.haemolyticus*	23	21	22	23	1.5	4	0.5/9.5	<0.125/4

Serial number	Phenotypic identification tests					Organism I.D.	Disk diffusion test results (mm)				E-test MIC results (μg/ml)		Agar dilution MIC results (μg/ml)	
	Acid from glucose	Citrate utilisation	Growth at 35°C	Growth at 44°C	Haemolysis on sheep blood agar		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
116	+	+	+	+	-	*A.baumanii*	19	17	26	19	16	16	0.13/2.38	128/4
117	+	+	+	+	-	*A.baumanii*	24	22	27	22	3	2	0.13/2.38	0.25/4
118	+	+	+	NG	+	*A.haemolyticus*	24	22	22	20	1.5	4	0.25/4.75	2/4
119	+	+	+	+	-	*A.baumanii*	15	17	0	18	>32	12	32/608	64/4
120	-	+	+	NG	-	*A.junii*	9	0	29	0	>32	>256	0.25/4.75	128/4
121	+	+	+	+	-	*A.baumanii*	16	17	0	16	24	12	32/608	64/4
122	+	+	+	+	-	*A.baumanii*	13	14	0	17	>32	32	8/152	32/4
123	+	+	+	+	-	*A.baumanii*	15	15	0	20	>32	24	>32/608	64/4
124	+	+	+	+	-	*A.baumanii*	17	15	0	21	>32	32	>32/608	128/4
125	+	+	+	+	-	*A.baumanii*	16	18	0	20	>32	16	4/76	32/4
126	+	+	+	+	-	*A.baumanii*	12	19	22	26	>32	6	0.06/1.19	<0.125/4
127	+	+	+	+	-	*A.baumanii*	23	22	25	26	1.5	3	0.06/1.19	<0.125/4
128	+	+	+	+	-	*A.baumanii*	15	17	0	21	>32	16	4/76	32/4
129	+	+	+	+	-	*A.baumanii*	15	18	0	17	>32	8	>32/608	64/4
130	+	+	+	+	-	*A.baumanii*	13	16	0	18	>32	16	>32/608	64/4
131	-	+	+	+	-	*A.baumanii*	12	21	21	25	>32	4	0.06/1.19	<0.125/4
132	+	+	+	+	-	*A.baumanii*	23	22	22	25	1.5	2	0.13/2.38	1/4
133	+	+	+	+	-	*A.baumanii*	23	22	0	26	1.0	2	4/76	<0.125/4
134	+	+	+	+	-	*A.baumanii*	15	16	0	18	24	16	>32/608	64/4
135	+	+	+	+	-	*A.baumanii*	20	21	22	24	2	2	0.06/1.19	<0.125/4
136	+	+	+	+	-	*A.baumanii*	14	11	0	14	>32	64	>32/608	512/4
137	+	+	+	+	-	*A.baumanii*	22	22	25	25	2	3	0.06/1.19	<0.125/4
138	+	+	+	+	-	*A.baumanii*	15	16	0	18	>32	12	>32/608	128/4
139	-	-	+	-	-	*A.lwoffii*	26	24	28	28	0.5	2	0.06/1.19	<0.125/4
140	-	-	+	-	-	*A.lwoffii*	23	19	0	23	0.5	4	8/152	<0.125/4
141	+	+	+	+	-	*A.baumanii*	17	16	0	21	32	16	>32/608	128/4
142	+	+	+	+	-	*A.baumanii*	15	15	0	20	>32	24	>32/608	64/4
143	-	-	+	-	+	*A.haemolyticus*	30	29	24	32	0.38	0.75	0.25/4.75	<0.125/4
144	+	+	+	+	-	*A.baumanii*	26	25	27	26	0.50	1.0	0.06/1.19	<0.125/4

Serial number	Phenotypic identification tests					Organism I.D.	Disk diffusion test results (mm)				E-test MIC results (μg/ml)		Agar dilution MIC results (μg/ml)	
	Acid from glucose	Citrate utilisation	Growth at 35°C	Growth at 44°C	Haemolysis on sheep blood agar		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
145	+	+	+	+	-	*A.baumanii*	12	9	0	12	>32	64	>32/608	>512/4
146	+	+	+	+	-	*A.baumanii*	15	16	0	18	>32	12	>32/608	128/4
147	+	+	+	+	-	*A.baumanii*	14	13	0	20	>32	24	>32/608	128/4
148	+	+	+	+	-	*A.baumanii*	14	14	0	21	>32	24	>32608	64/4
149	+	+	+	+	-	*A.baumanii*	16	16	0	20	32	12	4/76	32/4
150	+	+	+	+	-	*A.baumanii*	22	22	0	25	2	3	16/304	4/4
151	+	+	+	+	-	*A.baumanii*	22	22	26	24	2	3	0.13/2.38	0.25/4
152	+	+	+	+	-	*A.baumanii*	23	22	26	27	3	3	0.06/1.19	<0.125/4
153	+	+	+	+	-	*A.baumanii*	20	19	0	18	8	8	>32/608	32/4
154	+	+	+	+	-	*A.baumanii*	23	23	25	25	1.5	2	0.06/1.19	<0.125/4
155	+	+	+	+	-	*A.baumanii*	22	22	25	26	3	3	0.06/1.19	<0.125/4
156	+	+	+	+	-	*A.baumanii*	16	18	21	17	>32	12	0.25/4.75	64/4
157	+	+	+	+	-	*A.baumanii*	15	13	0	21	>32	24	>32608	64/4
158	+	+	+	+	-	*A.baumanii*	15	16	0	22	>32	16	4/76	32/4
159	+	+	+	+	-	*A.baumanii*	18	16	0	21	32	16	4/76	32/4
160	+	+	+	+	-	*A.baumanii*	17	16	0	22	>32	16	4/76	32/4
161	+	+	+	+	-	*A.baumanii*	22	20	25	23	3	6	0.06/1.19	1/4
162	+	+	+	+	-	*A.baumanii*	16	16	0	18	>32	16	32/608	128/4
163	+	+	+	+	-	*A.baumanii*	16	15	0	18	>32	16	>32/608	128/4
164	+	+	+	+	-	*A.baumanii*	23	20	27	23	0.38	3	0.03/0.59	<0.125/4
165	+	+	+	+	-	*A.baumanii*	18	13	0	24	32	32	4/76	32/4
166	+	+	+	+	-	*A.baumanii*	16	15	0	19	>32	24	>32/608	128/4
167	+	+	+	+	-	*A.baumanii*	21	20	0	23	4	4	16/304	16/4
168	+	+	+	+	-	*A.baumanii*	16	15	0	19	>32	24	>32/608	128/4
169	+	+	+	+	-	*A.baumanii*	15	16	0	19	>32	24	>32/608	256/4
170	+	+	+	+	-	*A.baumanii*	24	22	0	24	3	3	16/304	16/4
171	+	+	+	+	-	*A.baumanii*	25	23	27	25	0.75	2	0.03/0.59	<0.125/4
172	+	+	+	+	-	*A.baumanii*	18	18	17	17	>32	12	0.5/9.5	64/4

Table 4 The NCCLS acceptable quality control ranges of MIC (μg/ml) for the reference strains used in this study.

Antimicrobial agent	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Escherichia coli* ATCC 35218
CPIM	0.015 - 0.06	1 - 4	-
CTAZ	0.06 – 0.5	1 - 4	-
TMP-SULFA (1/19)	≤ 0.5/9.5	8/152 – 32/608	-
PIP-TAZO	1/4 - 4/4	1/4 – 8/4	0.54/4 – 2/4

Table 5 The NCCLS control limits for monitoring antimicrobial disk susceptibility tests, zone diameter (mm) limits for individual tests on Mueller-Hinton agar for the reference strains used in this study.

Antimicrobial agent	Disk content (μg)	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Escherichia coli* ATCC 35218
CPIM	30	29-35	24-30	-
CTAZ	30	25-32	22-29	-
TMP-SULFA	1.25/23.75	24-32	-	-
PIP-TAZO	100/10	24-30	25-33	24-30

Table 6. Results of the control reference strains

Run number	Reference strain	Zone diameter Disk diffusion test (mm)				MIC (µg/ml)			
						E-test		Agar dilution test	
		CPIM	CTAZ	TMP-SULFA	PIP-TAZO	CPIM	CTAZ	TMP-SULFA	PIP-TAZO
Run # 1	*Escherichia coli* **ATCC 25922**	35	31	26	28	0.047	0.25	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	31	-	-	-	4/4
	Pseudomonas aeruginosa **ATCC 27853**	27	29	0	29	3	1.5	-	-
Run #2	*Escherichia coli* **ATCC 25922**	36	31	26	29	0.032	0.125	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	32	-	-	-	1/4
	Pseudomonas aeruginosa **ATCC 27853**	28	29	0	29	3	1.5	-	-
Run #3	*Escherichia coli* **ATCC 25922**	36	32	26	29	0.032	0.125	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	31	-	-	-	1/4
	Pseudomonas aeruginosa **ATCC 27853**	28	28	0	29	2	1.5	-	-
Run # 4	*Escherichia coli* **ATCC 25922**	34	31	26	28	0.032	0.125	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	30	-	-	-	0.5/4
	Pseudomonas aeruginosa **ATCC 27853**	28	31	0	31	2	1.5	-	-
Run # 5	*Escherichia coli* **ATCC 25922**	35	31	27	28	0.032	0.125	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	31	-	-	-	1/4
	Pseudomonas aeruginosa **ATCC 27853**	26	29	0	30	2	1.5	-	-
Run # 6	*Escherichia coli* **ATCC 25922**	35	30	25	26	0.032	0.125	0.25/4.75	2/4
	Escherichia coli **ATCC 35218**	-	-	-	28	-	-	-	2/4
	Pseudomonas aeruginosa **ATCC 27853**	26	30	0	29	2	1.5	-	-
Run # 7	*Escherichia coli* **ATCC 25922**	35	32	28	28	0.032	0.125	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	30	-	-	-	1/4
	Pseudomonas aeruginosa **ATCC 27853**	27	29	0	31	2	1	-	-
Run # 8	*Escherichia coli* **ATCC 25922**	34	30	27	27	0.032	0.19	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	31	-	-	-	1/4
	Pseudomonas aeruginosa **ATCC 27853**	26	29	0	26	2	1.5	-	-
Run # 9	*Escherichia coli* **ATCC 25922**	35	31	27	29	0.047	0.125	0.13/2.38	2/4
	Escherichia coli **ATCC 35218**	-	-	-	31	-	-	-	1/4
	Pseudomonas aeruginosa **ATCC 27853**	28	29	0	30	2	1.5	-	-